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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The objective of this contract was to establish the feasibility of studying proteins on surfaces by scanning tunneling microscopy (STM) and atomic force microscopy (AFM). Amino acids and proteins deposited on highly oriented pyrolytic graphite (HOPG) were viewed by STM (Appendices A-C). A preliminary experiment was conducted in the laboratory of Dr. Paul Hansma, University of California, Santa Barbara, which demonstrated the observation of Immunoglobulin (IgG) on quartz and mica surfaces. An underwater real time observation of IgG adsorption on mica (Appendix D) suggests that the process is not homogeneous. We now have a functioning AFM in our lab - (thanks to the generosity and assistance of Paul Hansma and co-workers) - and protein imaging studies are in progress. <i>Keywords!</i>			

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Characterization of Biopolymer Surfaces Using Scanning Microscopies:

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Summary:

The objective of this contract was to establish the feasibility of studying proteins on surfaces by scanning tunneling microscopy (STM) and atomic force microscopy (AFM). Amino acids and proteins deposited on highly oriented pyrolytic graphite (HOPG) were viewed by STM (Appendices A-C). A preliminary experiment was conducted in the laboratory of Dr. Paul Hansma, University of California, Santa Barbara, which demonstrated the observation of immunoglobulin (IgG) on quartz and mica surfaces. An underwater real time observation of IgG adsorption on mica (Appendix D) suggests that the process is not homogeneous. We now have a functioning AFM in our lab - (thanks to the generosity and assistance of Paul Hansma and co-workers) - and protein imaging studies are in progress.

Publications:

Appendix A:

"Scanning Tunneling Microscopic Images of Amino Acids," L. Feng, C-Z Hu, J.D. Andrade, J. Microscopy 152 (1988) 811-816.

This paper presented images of amino acids adsorbed on HOPG. The STM was operated in air. Although individual amino acids were occasionally observed, the majority of adsorbates were dimers or clusters.

Appendix B:

"Scanning Tunneling Microscopic Images of Adsorbed Serum Albumin on HOPG," L. Feng, C-Z Hu, J.D. Andrade, J. Colloid Interface Sci. 126 (1988) 650-653.

Serum albumin adsorbed on HOPG from buffer solutions was observed by STM in air. The images show details of subdomains with a resolution of better than 20 Å. The image agrees with the suggested 3-D structure of albumin and with a recently reported X-ray crystal structure.

Appendix C:

"Scanning Tunneling Microscopy of Proteins on Graphite Surfaces," L. Feng, J.D. Andrade, C-Z Hu, Scanning Microscopy 3 (1989) 399-410.

This paper presents all of the biomolecule STM work done by our group through April, 1989. In addition to reviewing the results of the two papers discussed above, it presents results and images for lysozyme and fibrinogen on HOPG. The role of substrate binding affinity, tip-induced deformation, and motions and pile-up due to scanning processes are briefly discussed.

Appendix D:

"Direct Observations of Immunoglobulin Adsorption Dynamics Using the Atomic Force Microscope," J-N Lin, B. Drake, A.S. Lea, P.K. Hansma, J.D. Andrade, Langmuir (1989) in press.

The paper presents a portion of the results of an experiment done in Paul Hansma's laboratory at the University of California in Santa Barbara. An antiluorescein monoclonal antibody was adsorbed from dilute solution onto clean mica. The adsorption appeared to result in aggregates or clusters. Although individual adsorption events (collisions) could be seen, the molecules were not stable and apparently desorbed readily. This suggests that we may have to treat IgG adsorption as a highly cooperative, nonrandom process. Based on this work we proceeded, with the generosity and assistance of Hansma and his group, to build an AFM in our laboratory, which is now functioning (as of Dec. 1, 1989).

Appendix E:

"Real Time Imaging of Immunoglobulin Adsorption on Mica Using the Atomic Force Microscope," J-N Lin, B. Drake, A.S. Lea, P.K. Hansma, and J.D. Andrade, abstract submitted for 16th Annual Meeting of the Society for Biomaterials, May 20-23, 1990, Charleston, South Carolina.

Basically the same as Appendix D.

Scanning tunnelling microscopic images of amino acids

by L. FENG, C. Z. HU and J. D. ANDRADE*, *Department of Bioengineering and Center for Biopolymers at Interfaces, University of Utah, Salt Lake City, Utah 84112, U.S.A.*

KEY WORDS. Amino acids, adsorption, graphite, charge transfer.

SUMMARY

We present images of amino acids adsorbed on highly orientated pyrolytic graphite (HOPG) obtained with the scanning tunnelling microscope (STM) in air. Individual molecules can be observed although the majority of adsorbates appear to form clusters. In the case of leucine, methionine, and tryptophan, two molecules often associate together to form a dimer. Single or dimer glycine molecules were not seen, but a cluster of a number of them was observed. The various adsorbed states may be related to the different interactions between the amino acids and the graphite surface. The mechanism of image formation of the amino acids is probably related to charge transfer mechanisms.

The scanning tunnelling microscope (STM) has been used for studies of a number of organic and biological substances, including copper phthalocyanine Gimzewski *et al.* (1987), sorbic acid, Smith (1987), bacteriophage, Baro *et al.* (1985), DNA, Travaglini *et al.* (1987) and Lindsay & Barris (1988), proteins, Dahn *et al.* (1988), and lipid bilayer, Smith *et al.* (1987) on various substrates. The results have indicated that the STM may be applied in biology owing to its high resolution, ambient and under-liquid working conditions, and easy operation. Here we report the STM observation of four amino acids adsorbed on highly orientated pyrolytic graphite (HOPG).

The STM was provided by the Tunneling Microscope Co. based on the D. Smith design, Smith (1987). The HOPG, from Union Carbide, was readily peeled with a tape. Tryptophan (trp) was from Calbiochem-Behring Co. and glycine (gly), leucine (leu), and methionine (met) were from Sigma Chemical Co. Their chemical structures and planar dimensions based on the CPK[®] atomic models (Ealing Corp.) are shown in Fig. 1. Their aqueous solutions were prepared by dissolving amino acids in pure water (10 MΩ/cm). The concentrations were all 0.1 mg/ml for the STM measurement. A drop of the solution was placed on a freshly cleaved HOPG surface for 5 min before the surface was flushed with ultra-pure water for a very short time. The adsorbed specimens were dried at room temperature and normal pressure for at least overnight before the image was taken. The STM study was performed in air. Constant height mode was used as it gave better resolution and less distorted images than the constant current mode, Smith (1987). In this mode the gap distance is held invariant, and the tunnelling current

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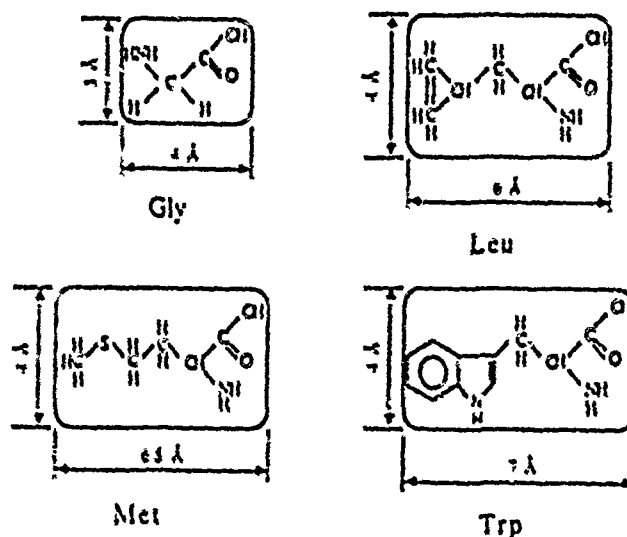


Fig. 1. Chemical structures and molecular sizes, estimated from the CPK[®] atomic models, for the four amino acids.

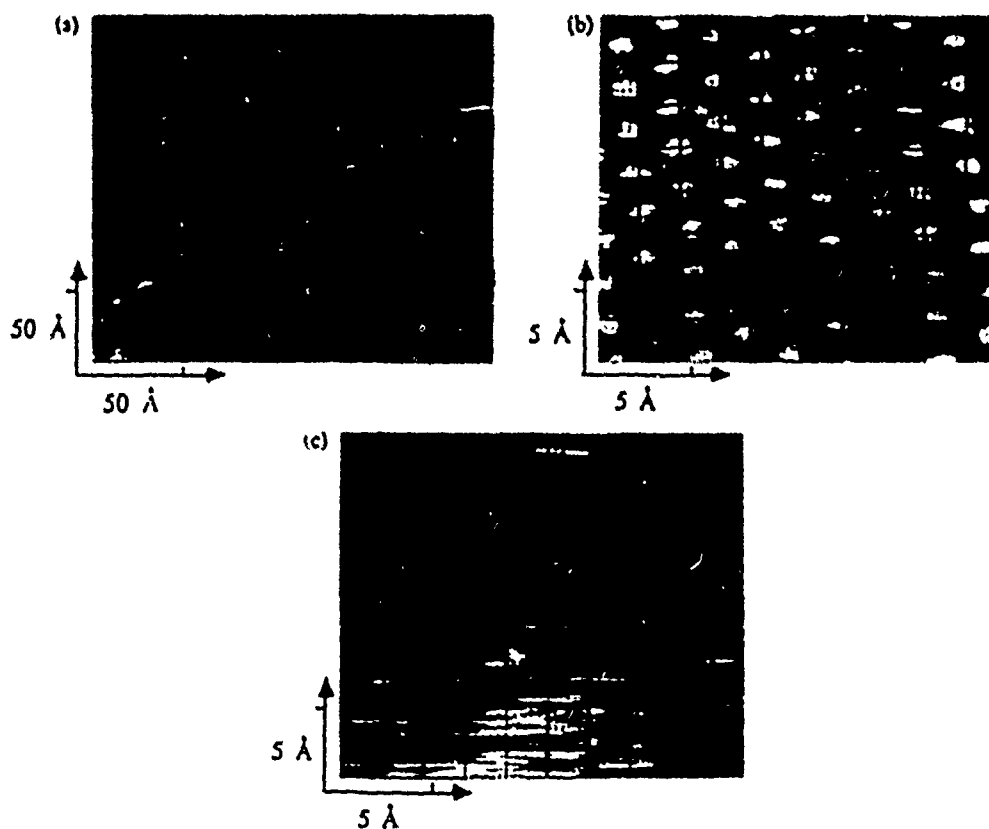


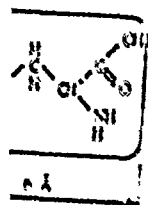
Fig. 2. Images of deposited trp. (a) Low magnification image. (b) High magnification graphitic image of the upper left part of (a). (c) High magnification trp image of the lower right part of (a).

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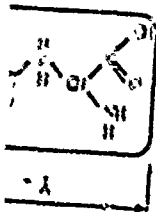
In order to obtain a large amount of data in two rows divided into two times, the images were taken. The lower resolution pictures were much rougher than they represent the adsorbate layer. The change in the number of molecules has been described thought to be with the aid of Bixon, 1987) could raise the number of current via c.

The major routine observations were observed suggest that 1 of the four species from the most frequently observed

Fig. 3. Gly image. A number of molecules



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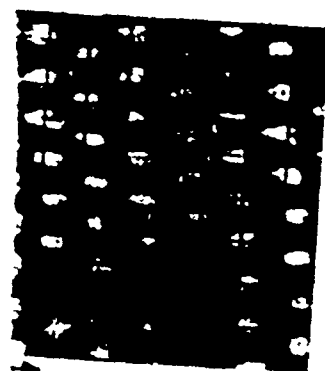
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atomic models, for the four amino

changes according to the surface contour and composition during x-y scanning of the tip. It is this change that provides information on the adsorbed species. The tungsten tip was electrochemically etched in a 2 M KOH aqueous solution (Smith, 1987). Operating parameters included 1–2 nA tunnelling current, 0.2–0.8 V bias voltage, and 1 kHz scan rate in X direction and 1 Hz in Y direction. The images were displayed by a grey-scale oscilloscope and the pictures were taken by a CRT camera.

In order to assure that amino acid molecules could be observed under the STM, a relative large amount of trp was deposited on HOPG without flushing. Figure 2(a) shows that there are two rows dividing the picture into two parts. When the magnification was increased to ten times, the image of the upper left part, Fig. 2(b) is that characteristic of a typical HOPG surface. The lower right part, Fig. 2(c) shows no graphitic character, however, and the surface was much rougher. Here the current variation reached 5 nA during scanning. Since these two pictures were taken under identical conditions and almost simultaneously, it is thought that they represent two different surface states. The latter, Fig. 2(c) image, should be of the adsorbate layer and the rows in Fig. 2(a) are presumably its edge. Therefore, amino acids can change the tunnelling current and can be detected by the STM. The principle of the STM has been described in detail, Hansma & Tersoff (1987). Amino acids and proteins have long been thought to be semiconductors since charge transfer can occur between their functional groups with the aid of impurities, such as metal ions or water (Gutmann & Lyons, 1967; Jortner & Bixon, 1987); particularly, sorbed water may play a very important role (Panitz, 1987). Water could 'raise the dielectric constant and therefore stabilize the electron-hole pair, thus increasing the number of charge carriers', (Eley & Leslie, 1963), which would enhance the tunnelling current via charge transfer mechanisms.

The majority of the images showed segregated clusters. The non-uniform distribution was a usual observation in these studies. Despite their rare occurrence some individual molecules were observed and they are clearly shown in Figs. 3–6 for each of the four amino acids. We suggest that the bright humps represent the amino acid molecules because: (a) the dimensions of the four species, estimated with reference to the graphitic crystal lattice, agree with those from the molecular models (see the insertions in each figure); (b) the 'bright humps' were frequently observed for the adsorbed samples but similar images were not seen on clean HOPG



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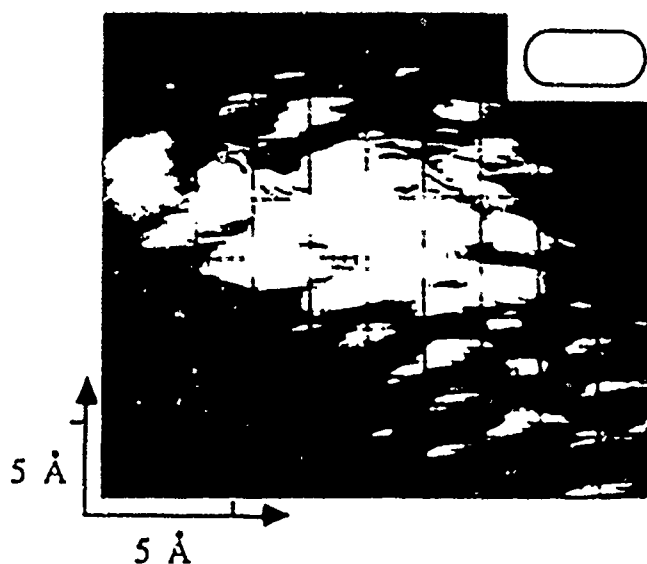


Fig. 3. Gly image. The insertion is the molecular size, based on CPK models, of the same magnification as the image. A number of gly molecules apparently pack together. The distance in between is about 3 Å.

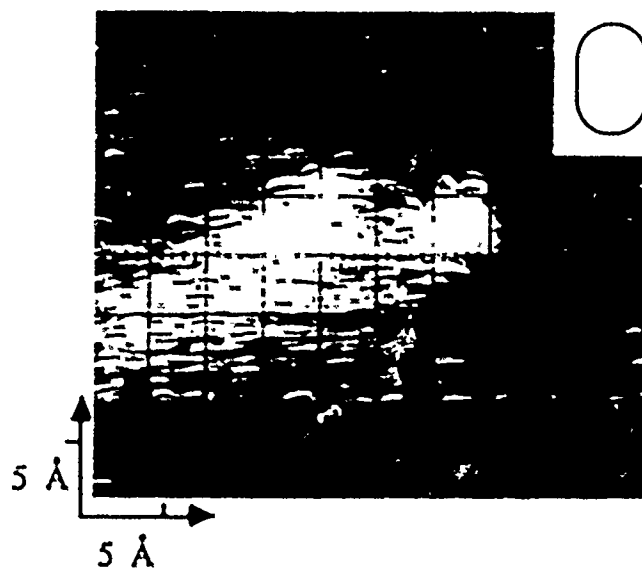


Fig. 4. Trp image. The insertion is the molecular size, based on CPK models, of the same magnification as the image. A dimer of two parallel packed molecules can be seen. The distance in between is about 9 Å.

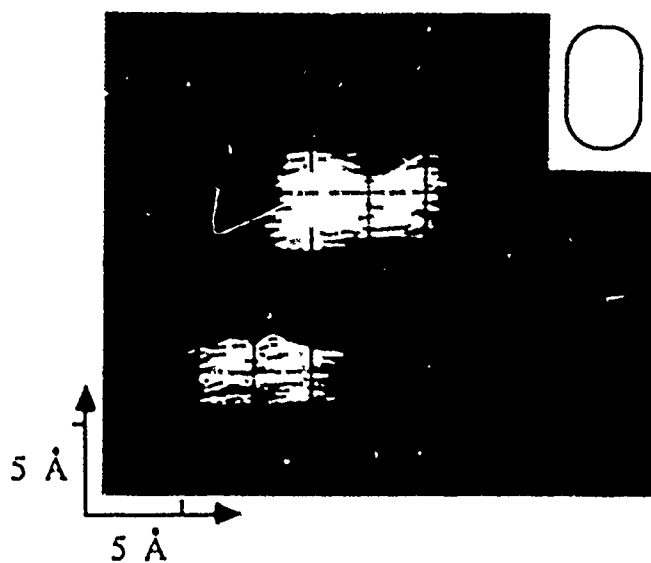


Fig. 5. Leu image. The insertion is the molecular size, based on CPK models, of the same magnification as the image. Three leu molecules can be seen, two of which are associated to form a dimer. The distance between the two is about 5 Å.

surfaces or on a control sample which had undergone identical sample preparation procedures except for no amino acids; (c) taking pictures of the bright humps proved to be difficult since they tended to escape very easily due to their weak interactions with the substrate; (d) adsorption from the atmosphere was not given strong consideration because graphite surface images were routinely obtained on 'clean' HOPG, even samples used several days after cleavage; (e) hydrocarbon impurities, if any, are not normally seen by STM, (Schneir &



Fig. 6. Met image. The image quality is poor. One met molecule is visible.

Hansma, 1987); and (f) events.

It is interesting to amino acid, gly, form. Another characteristic were also observed with gly. In fact it was very difficult to see a dimer was not a doubt thought there are two pairwise 'side-on' associations may remain intact a molecule does not have a very high probability of being quite volatile under the STM observation. A sort of surface defect amino acids require is why we only saw

At this time the out and further work. Although we have analysis of amino potential for surface

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We thank Prof. Smith for assistance in the donation of the high-resolution Biopolymers at In



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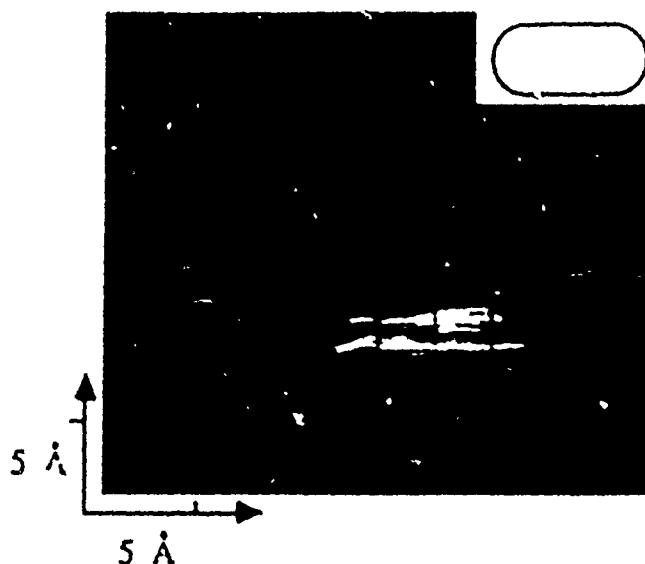


Fig. 6. Met image. The insertion is the molecular size, based on CPK models, of the same magnification as the image. One met molecule is lying horizontally on HOPG. Dimers were often observed but the pictures are poor in quality.

Hansma, 1987); and (f) all the pictures in this paper are representative of many observation events.

It is interesting to look at the molecular packing of the different adsorbates. The simplest amino acid, gly, formed arrays, each of which contains a number of gly molecules (Fig. 3). Another characteristic is the dimer aggregation for both trp and leu (Figs. 4 and 5). Dimers were also observed with met. The dimer or trimer phenomenon was not uncommon except for gly. In fact it was very difficult to spot a single separated molecule, such as met in Fig. 6. A dimer was not a double tip artefact because not everything on the image was doubled. It is thought there are two reasons for the dimer or multimer formation. Amino acids can form pairwise 'side-on' associations in their aqueous solutions, (Lilley, 1985). The dimer association may remain intact during the adsorption process. On the other hand, a single amino acid molecule does not have a strong interaction with the HOPG surface. Without the association, it would be quite volatile due to its low molecular weight. This explains its observed mobility under the STM observation. That is why we often located one single molecule but it soon disappeared before we could take a picture. Those that were imaged may have adhered to some sort of surface defect, which could increase adsorbent-adsorbate interactions. The smaller amino acids require more intermolecular interactions in order to form a stable adsorbed state. It is why we only saw gly clusters rather than dimers.

At this time the possibility of selective adsorption of impurities cannot be completely ruled out and further work is necessary, such as scanning tunnelling spectroscopy, (Smith, 1987). Although we have much more to observe and to learn about electron tunnelling and STM analysis of amino acids and larger biomolecules, it is clear that STM offers considerable potential for surface studies of biomolecules.

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We thank Professor C. F. Quate for stimulating our interest and activity on STM and Dr D. Smith for assistance with the STM. We also thank Dr Moore of the Union Carbide Corp. for the donation of the highly orientated pyrolytic graphite. This work was funded by the Center for Biopolymers at Interfaces, University of Utah.

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LETTER TO THE EDITOR

Scanning Tunneling Microscopic Images of Adsorbed Serum Albumin on Highly Oriented Pyrolytic Graphite

Human serum albumin molecules, adsorbed on highly oriented pyrolytic graphite (HOPG), have been observed by scanning tunneling microscopy (STM) in air. The images show the details of sub-molecular domains as well as individual molecules with a resolution better than 20 Å. The observed domain arrangement agrees with the expected size and structures of albumin, suggesting that little denaturation has occurred on HOPG. This work further demonstrates that the direct observation of biomolecules by STM is probable. © 1988 Academic Press, Inc.

Scanning tunneling microscopy (STM) has enabled scientists to probe structure and topography at the Angstrom level. STM has been successfully applied to conducting and semiconducting materials (1, 2). Application to biomaterials, however, seems to be a controversial subject. Adsorbed biological substances are normally insulators and are generally mobile. Thus they are difficult to "see" by STM. Although such difficulties can be overcome by surface coating with conducting materials (3, 4), the direct observation of biomaterials is more attractive, as direct observation minimizes artifacts and makes *in situ* measurements possible. A few published papers have given images of bacteriophage (5), DNA (6, 7), lipid bilayers (8), and proteins (9, 10) though the image formation mechanism remains unexplained. Our STM experience suggests that biological substances can be sensed by STM, depending on the type of molecular species, its adsorbed state, its hydration degree, the tip shape, the stability of the STM instrument, etc. In this report we present two STM pictures of protein molecules adsorbed on highly oriented pyrolytic graphite (HOPG). The images reveal the best resolution so far achieved by STM on proteins. The result unambiguously indicates the feasibility of direct observation of biomacromolecules with STM.

Human serum albumin has a concentration of about 42 g/liter in plasma and constitutes 60% of the mass of plasma proteins (11). It is a single polypeptide chain consisting of 584 amino acid residues and having a molecular weight of about 69,000. Albumin has a strong internal structure, held firmly together by 17 disulfide bridges. Figure 1 shows the three-dimensional molecular model. The molecular shape is generally taken as an ellipsoid with dimensions of 40 × 140 Å. There are three domains within the molecule. The domain structure is believed to be a cylinder formed by six α -helices (11).

The STM images of human albumin are presented in Fig. 2. Figure 2a shows one adsorbed human albumin molecule and some parts of two other molecules on HOPG. They have different orientations. The molecular dimensions are 120 Å in length and 60 Å in width. The slight

deviation in dimensions of the adsorbate from those of the above model may be caused by slight collapse of the native structure in the relatively dry air environment. Three cylindrical, parallel domains can be observed, as expected from the model, suggesting that the surface denaturation is not extensive. In addition to domains, some side loops connecting the domains can also be seen. The fact that domains can be distinguished means that the resolution is about 10 Å. The flat regions around the adsorbed molecules have been identified as bare HOPG. In fact, the tiny ripples along the scanning lines in Fig. 2b are the corrugation of graphitic carbon atoms, commonly observed by STM on HOPG (1). The surface depression to the left of each adsorbed molecule is considered an influence of the adsorbed species. This area should actually be the flat substrate, which can be confirmed by the existence of the atomic corrugations in that region. Similar observations have been reported (12).

Figure 2 gives information on adsorption as well. Certain carbon materials have long been considered to have excellent biocompatibility (13, 14). One of the hypotheses is that there is not much denaturation of proteins adsorbed on a carbon surface. Although HOPG is not quite the same as those carbon materials, its adsorbing behavior may still imply that this hypothesis is reasonable. The albumin molecules essentially kept their native state in the presence of interactions between the adsorbate and the adsorbent. From this respect it is worth studying the adsorption under water in the future.

In summary, we have been able to obtain STM images of albumin and of its domain structure on HOPG. This work confirms that STM can be employed to study some biological substances under certain experimental conditions, such as for protein adsorption on conducting substrates. We have observed several different proteins deposited on HOPG. We consider that the image formation mechanism is related to charge transfer processes in the protein (15). By the aid of scanning tunneling spectroscopy (STS) (16), STM might become a powerful tool to study the electronic properties of proteins.

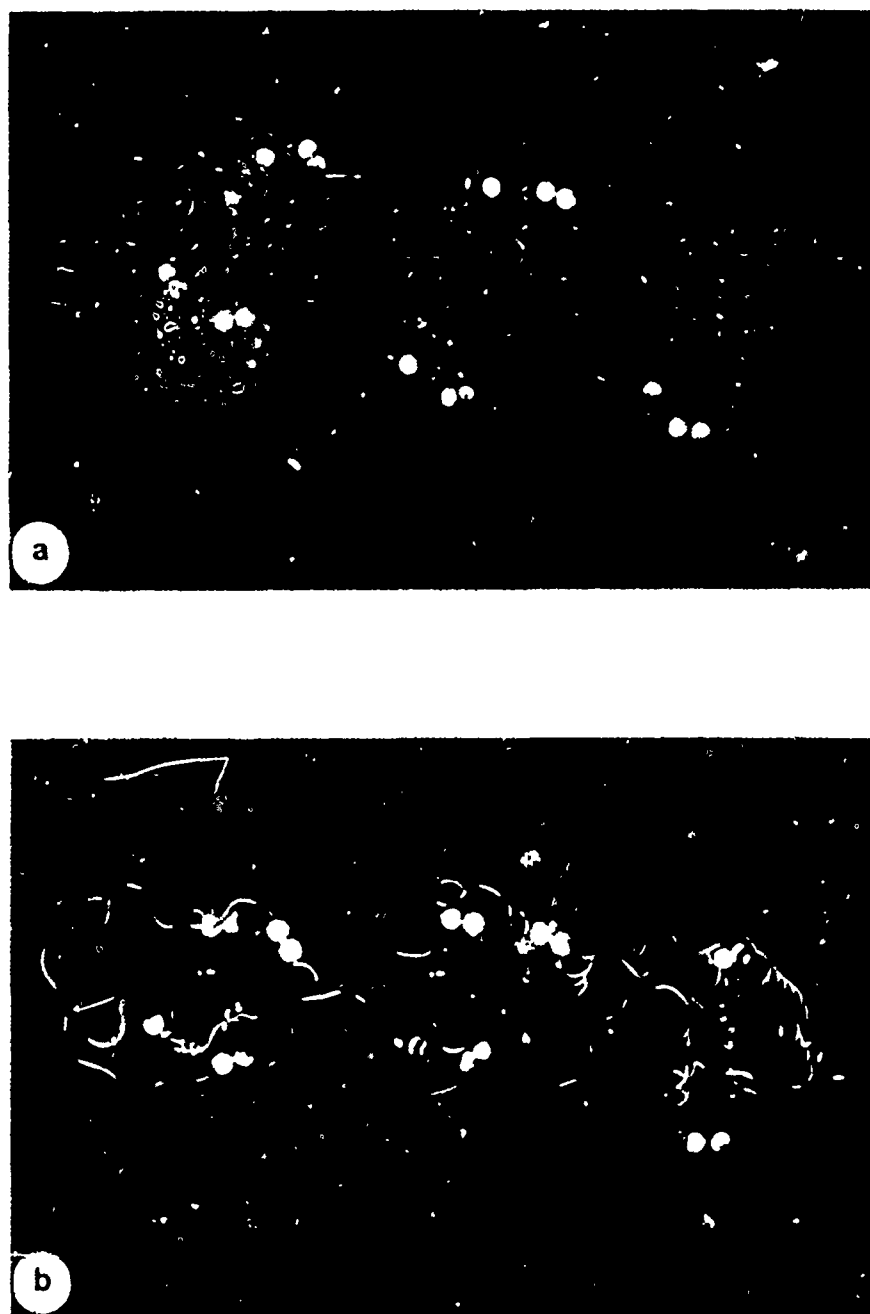


FIG. 1. Backbone three-dimensional model of serum albumin. In this model the wire represents the peptide backbone and the spheres represent disulfide bridges in the long loops. (a) Side view of the model. The three domains are antiparallel to one another. (b) Top view of the same model. The length is 140 Å and the width 40 Å for the entire molecule (from Ref. (11) by permission of the author).

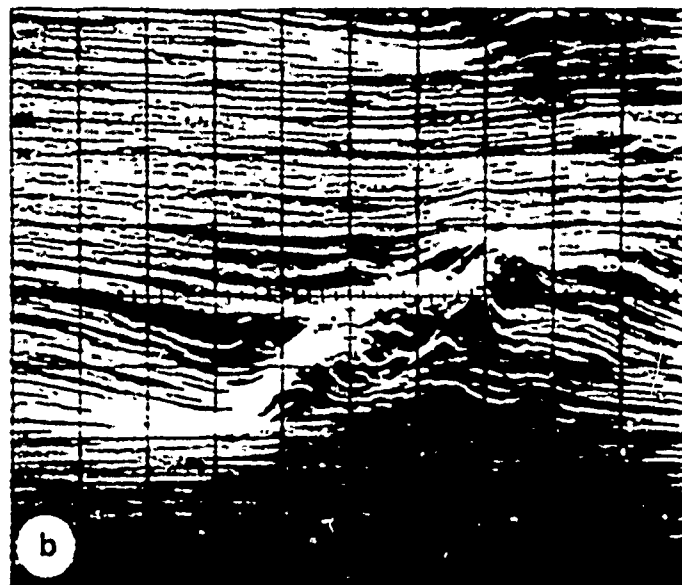
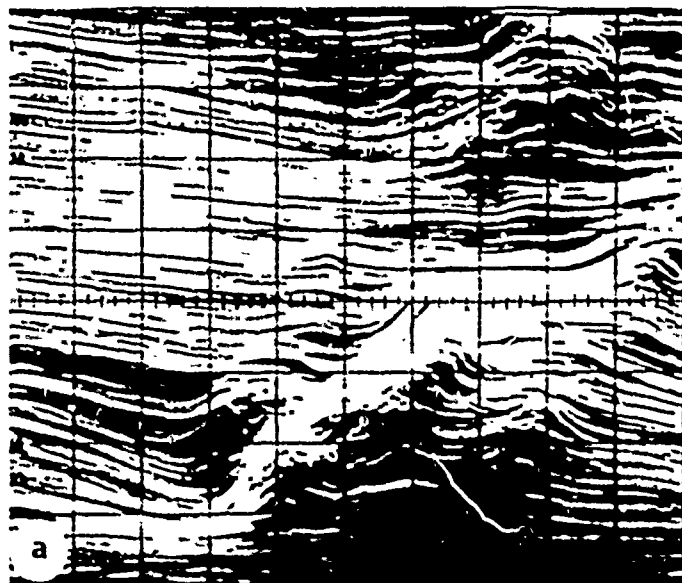


FIG. 2. Images of human albumin on HOPG. The picture dimensions are 200 \AA in the horizontal (x) and 160 \AA in the vertical (y) directions. Since the adsorbate has a very different electronic structure from the substrate, the height (z direction) could not be directly measured. Both (a) and (b) show several different molecules. Three domains can be clearly seen (a). The ripples on the raster lines in (b) are the corrugations of carbon atoms of the HOPG. The sample was prepared by depositing a droplet of albumin (Calbiochem) in phosphate-buffered saline solution (pH 7.4, 10 ppm albumin) onto freshly cleaved HOPG and then the droplet was removed by capillarity with a tissue. The sample was then flushed with water for 10 s and was dried at room temperature for 5 h before observation. The STM was operated in air with a bias voltage of 200 mV, tunneling current of 4 nA, and high feedback gain. The tungsten tip was electrochemically etched in 2 M KOH. Constant height mode was used with the scanning rates 40 Hz in x and 0.05 Hz in y .

AC:

We thank Profes
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SCANNING TUNNELING MICROSCOPY OF PROTEINS ON GRAPHITE SURFACES

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Abstract

We applied scanning tunneling microscopy (STM) to the observation of amino acids and proteins deposited and/or adsorbed on highly oriented pyrolytic graphite (HOPG).

Although many questions remain, it is demonstrated that relatively high resolution images of uncoated proteins can often be obtained in air. We present images of five amino acids (glycine, leucine, lysine, methionine and tryptophan) and three proteins (lysozyme, albumin and fibrinogen) under various conditions of deposition and adsorption. We discuss the role of affinity of the amino acids and proteins to the substrate, their adsorbed states and distribution, and STM tip-induced deformation and/or destruction.

STM studies of adsorbed proteins are expected to provide useful and even unique information on the conformation and packing of the proteins.

Introduction

Scanning tunneling microscopy (STM) is a new and fast growing surface analysis and imaging technique. In the seven or so years since its invention by Binnig and Rohrer (Binnig et al., 1982), STM has been gradually increasing in popularity in the imaging of conducting and semi-conducting surfaces (Binnig and Rohrer, 1985, Quate, 1986, and Hansma and Tersoff, 1987). Such rapid progress is due to the unparalleled capabilities of STM compared with other forms of microscopy: (1) ultra-high resolution down to atomic dimensions, (2) three-dimensional images, especially with a very high sensitivity in the vertical direction, (3) a variety of operating conditions, including vacuum, air and even liquids, (4) observation range from 10^{-6} to 10^{-10} m, (5) the ability to do tunneling spectroscopy, and (6) relatively inexpensive equipment.

The operating principle of STM is surprisingly simple. When a metal needle-like probe (tip) is brought close enough to a conducting surface (1-10 Angstroms), electrons tunnel through the gap between the tip and the surface under an appropriate bias voltage, producing a tunneling current. The tunneling current is a function of the bias voltage and the shape of the barrier (related to work function) and is extremely sensitive to the gap distance. The tunneling current is changed by a factor of 10 when the distance changes just 1 Angstrom for a local work function of 4 eV. It is this strong distance dependence that is the reason for STM's high vertical resolution. When the tip is rastered across the surface using a piezo-scanner, a feedback network adjusts the height of the tip above the substrate surface to keep the tunneling current constant; this is called the constant current mode. Alternately, the change in the tunneling current can be recorded at a constant tip height: the constant height mode. In

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both modes a surface topographical map is obtained (Hansma and Tersoff, 1987), as in Figure 1, if the substrate has a chemically homogeneous surface. Suppose there is an adsorbed molecule on a conducting surface; it may perturb the magnitude of the local tunneling current due to a change in local work function. The molecule is "imaged" through the change of the local current. This is the probable mechanism by which an adsorbate is detected by STM (Panitz, 1987 and Spong et al. 1989). Since the adsorbate usually does not have the identical chemical composition and structure as the substrate, the tunneling current map does not necessarily represent the same surface topography.

Since our group has a strong involvement in the study of proteins at interfaces (Andrade, 1985), we have a particular interest in applying STM to this area. Our rationale is as follows: conventional TEM or SEM generally needs a high vacuum system, which often distorts the protein native state. Often a coating is necessary to minimize sample charging and to enhance the contrast; such coating can easily introduce artifacts and thereby decrease the useful resolution. Labeling adsorbed proteins with heavy metals, such as gold, does not give a sufficiently high resolution in the SEM, and it depends on labeling efficiency and other factors. However, with an in-air-operated STM it is possible to observe proteins in their hydrated state in a humid environment. It is even possible to see proteins in solution with an appropriately designed and constructed STM. Proteins are considered semiconductive in their usual hydrated state (Jortner and Bixon, 1987).

The resolution of STM for protein molecules should be higher than in SEM or TEM. So long as the substrate conducts electricity, STM may be employed.

A brief review of STM applications in biology

Listed here are the major obstacles in applying STM to the study of proteins. (1) They are, in general, poor conductors of electricity so that they may not significantly alter the tunneling current; (2) they are relatively soft and flexible so that they tend to "smear out" the image and lower the resolution because of their motion and relaxation in the presence of the tip and the applied electric field; (3) their molecular structure is often not well characterized so that image interpretation is difficult; and (4) they may have weak interactions with the conducting substrate to which they are attached so that they are often perturbed or moved by the moving tip. Nevertheless, many biological as well as organic substances in different forms have been observed by STM. A few review papers are now available (Hansma et al., 1988 and Zasadzinski, 1989).

The very first paper of STM images of a biological substance, DNA, appeared in 1983 (Binnig and Rohrer, 1983), unveiling the possible application of STM in biology. Baro et al. (1985, 1986) reported the surface topography of bacteriophage $\phi 29$ on graphite. There have been a number of papers on imaging Langmuir-Blodgett films on different substrates by STM, with arachidate on graphite (Smith et al., 1987), dimyristoylphosphoric acid on both

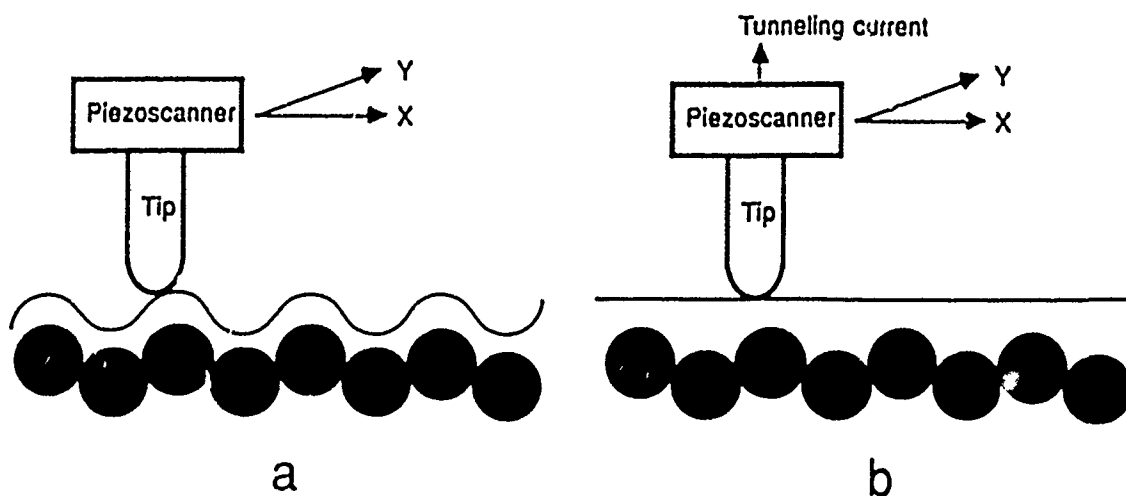


Figure 1. Schematic expression of operating principles of scanning tunneling microscopy in either the constant current mode and constant height mode.

graphite and gold (Horber et al., 1988), *o*-tricosanoic and 12,8-diynoic acids (Cd salts) on silicon wafer and graphite (Braun et al., 1988). It seems that with such regularly packed structures, the molecules are much easier to image and distinguish and the resolution is higher compared to individual or randomly packed molecules. The same is true when imaging liquid crystals (Foster and Frenner, 1988 and Speng et al., 1989) and TTF-TCNQ crystals (Sleauer and Tycko, 1988). Stemmer et al. (1987) have managed to image biological membranes (porin membrane). Membranes prepared by freeze-fracture replica methods show much more detail (Joseph et al., 1988). Studies of single stranded DNA have produced impressive results. Travaglini et al. (1987) started the study of bare DNA molecules. Later the same group obtained images of DNA by means of conducting film coatings (Amrein et al., 1988). Beebe et al. (1989) achieved a similar resolution on uncoated double-stranded DNA using STM. DNA images under water were obtained by Barris et al. (1988) and Lindsay et al. (1989).

Few papers have dealt with the subject of STM observation of proteins. One of the earliest papers on protein STM images was by Dahn et al. (1988). While their work was mainly on bacterial sheaths, a globular protein, ovalbumin, was imaged. The molecules had become flattened and elongated presumably due to the dehydration. Horber et al. (1988) studied Concanavalin A embedded in a lipid film. They claimed that the four subunits of Con A might be seen. Simic-Krstic et al. (1989) recently observed microtubules on graphite fixed in 0.1% glutaraldehyde in both freeze dried and hydrated states. Microtubules frequently appeared buckled, semiflattened and/or twisted. Collagen strands of 15 Angstroms in diameter on graphite were imaged by Voelker et al. (1988). They suggested that the periodic spikes from the strand represented pyrrolidine rings of the proline and hydroxyproline amino acid residues. In contrast to the DNA images, proteins on a conducting substrate generally show a less defined structure and poorer resolution.

Since protein adsorption properties play an important role in the applications of biomaterials, we believe that it is worthwhile to utilize STM to explore the details in conformation and packing of adsorbed proteins. STM may also provide information on the electronic structure of proteins which will certainly benefit molecular electronics studies. In the rest of the paper, we will introduce our published and unpublished STM work on five amino acids and three proteins (Feng et al., 1988 and 1989).

Experimenta

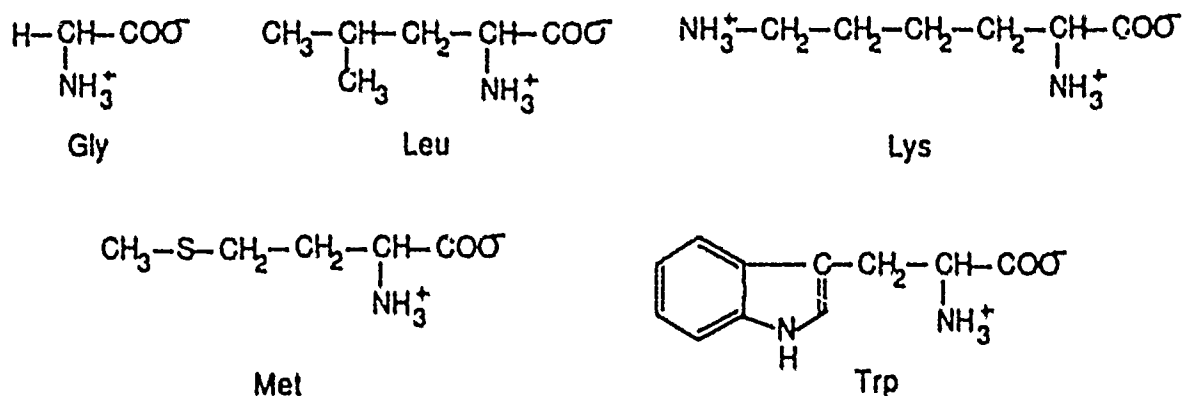
The substrate was highly oriented pyrolytic graphite (HOPG) from Union Carbide. As a routine substrate for STM, HOPG is a semimetal and relatively inert material. Cleaved by an adhesive tape, HOPG readily provides a large (1 mm x 1 mm) clean area with an atomically flat plane. Tryptophan (trp) was from Calbiochem and glycine (gly), leucine (leu), lysine (lys), and methionine (met) were from Sigma. Hen lysozyme was from Calbiochem, human serum albumin from Calbiochem and human fibrinogen from Calbiochem and Sigma. The amino acids and proteins were dissolved either in ultra-pure water (10 M Ω /cm) or in pH 7.4 phosphate buffered saline (PBS); amino acid concentrations were 0.1 mg/ml and protein concentrations were from 0.001 mg/ml (1 ppm) to 0.1 mg/ml (100 ppm). A droplet of the solution was pipetted onto a newly cleaved HOPG surface, which was either promptly removed by capillarity (for a deposited sample) or allowed to rest for 5 min before being flushed with ultra-pure water (for an adsorbed sample). All samples were dried at room temperature and ambient atmosphere (22°C and 20-50% R.H.).

Our STM was provided by the Tunneling Microscope Co. (Smith, 1987). STM tips were prepared by electrochemical etching a tungsten wire, 0.5 mm in diameter, in a 2 M KOH solution under a 20-30 V a. c. potential. The tips had diameters from 0.1-1 μ m at the end as measured by TEM. STM was operated in air; both constant height and constant current modes were used. Parameters for a typical constant height mode were 200-800 mV bias voltage (V_b), tips being negative with respect to samples, 1-2 nA tunneling current (I_t), 1 KHz scan rate in x direction and 1 Hz in y direction. Parameters for a typical constant current mode were 50-400 mV bias voltage, tips being negative with respect to samples, 1-4 nA tunneling current, 30 Hz scan rate in x direction and 0.05 Hz in y direction. The magnification was calibrated by the lattice parameters of HOPG substrate. Real-time images were processed by a band pass filter to minimize high frequency noise, and displayed by an oscilloscope. The pictures were recorded from the oscilloscope by a CRT camera.

Results and discussion

Amino acids (Feng et al., 1988)

We first studied the amino acids since they are the simpler building blocks of complex proteins. All five amino acids adsorbed on HOPG were easily seen as aggregates (Figure 2). Adsorbates occupied roughly 5-10% of



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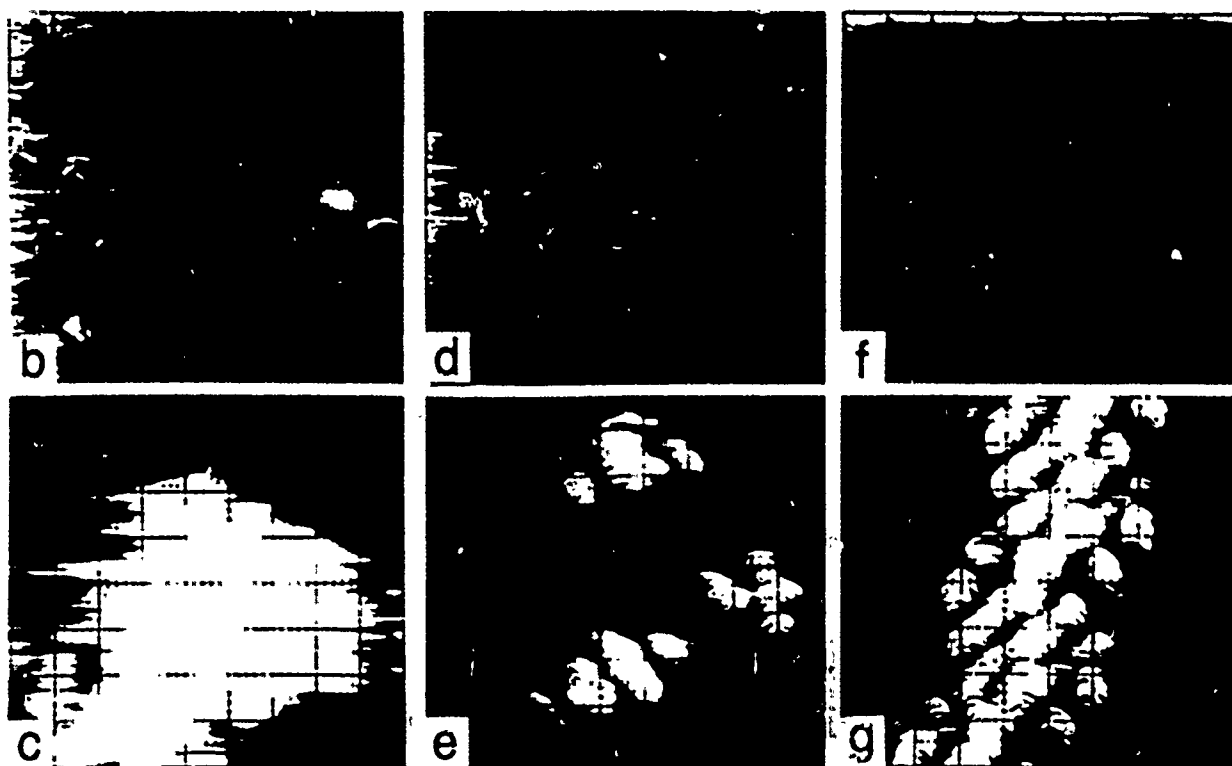


Figure 2. (a) Molecular formulas of the five amino acids. (b) - (g) Amino acids adsorbed from 0.1 mg/ml aqueous solutions on HOPG as aggregates. (b) Gly ($V_b = 500$ mV, $I_t = 1$ nA, 25 Angstroms/div): (c) One of the humps in (b), 2.5 Angstroms/div; (d) Lys ($V_b = 300$ mV, $I_t = 1$ nA, 25 Angstroms/div); the white vertical lines are photo defects; (e) Higher magnification of (d), 2.5 Angstroms/div; the white vertical lines are photo defects; (f) Met ($V_b = 800$ mV, $I_t = 1$ nA, 25 Angstroms/div); (g) Higher magnification of met ($V_b = 300$ mV, $I_t = 1.8$ nA, 2.5 Angstroms/div). In (c), (e) and (g), individual molecules can be barely seen. Constant height mode was used.

total surface, according to a statistical estimate with many regions. This value is much less than that expected from our radioisotope measurements, which gave about 80% coverage if monolayer adsorption was assumed. The discrepancy may be due to: (1) the presence of multilayer adsorbates which had been truncated by the STM tip because the gap distance was of the order of 1 Angstrom, (2) loss of adsorbates from the substrate when they were impounded by the rigid tip, and/or (3) incapability of imaging some adsorbates since they did not modify the tunneling current. From Figures 2 (c), (e) and (g) one could distinguish a few single molecules in those clusters. The apparent difference in their sizes is thought due to their different distances to the tip since they were randomly packed. More often than not, separate individual amino acid molecules were hard to find, presumably due to weak interactions between them and the substrate. Sometimes a molecule was spotted but it quickly disappeared from the image before a picture could be taken.

Occasionally, a few amino acid molecules were caught and imaged with better resolution, as in Figure 3. We suggest that the bright humps represent amino acid molecules because: (1) the dimensions of the three species, estimated with reference to the graphite crystal lattice, agree with those expected from the molecular models (see the insertions in each figure); (2) "bright humps" were frequently observed for the adsorbed samples but similar images were not seen on clean HOPG surfaces or on a control sample which had undergone identical sample preparation procedures except for no amino acids; (3) taking pictures of the bright humps turned out to be difficult since they tended to escape very easily due to their weak interactions with the substrate; (4) adsorption from the atmosphere was not given strong consideration because graphite surface images were routinely obtained on "clean" HOPG, even samples used several days after cleavage; (5) hydrocarbon impurities, if any, are not normally seen by STM, Schneir and Hansma(1987); and (6) all the pictures in this paper are representatives of many observation events. Dimers or trimers are relatively more stable than monomers in terms of interactions with the substrate so that they were immobile for a sufficient time for producing a photograph. No dimers or even trimers could be seen on gly samples because of gly's much smaller molecular weight and size.

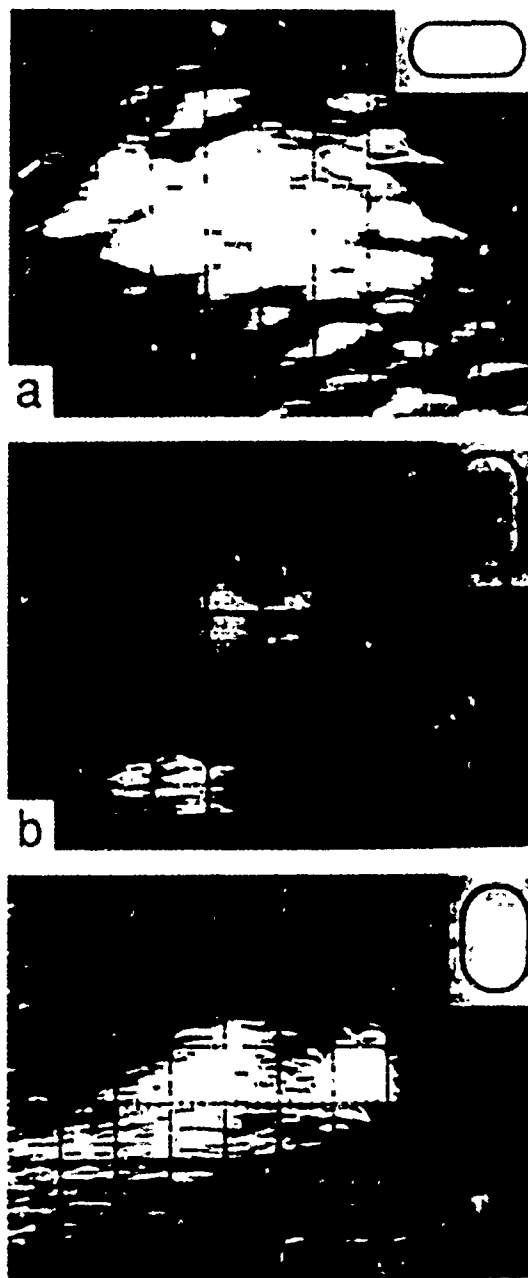


Figure 3. Images of individual amino acid molecules with constant height mode. The graphite substrate can be seen underneath. The insertions are the molecular sizes based on CPK models of the same magnification as the images. (a) Gly image (1.9 Angstroms/div in x and 3.2 Angstroms/div in y), a number of gly molecules apparently pack together; (b) Leu image (2.8 Angstroms/div in x and 3.2 Angstroms/div in y), three leu molecules being seen, two of which are associated to form a dimer; (c) Trp image (3.3 Angstroms/div in x and 3.8 Angstroms/div in y), a dimer of two parallel packed molecules being seen.

Hen egg-white lysozyme

Hen egg-white lysozyme is a small compact protein with molecular weight of 14,600, made up of a single polypeptide chain of 129 amino acids. Four disulfide bonds cross-link the molecule and provide high stability. Lysozyme has an ellipsoidal shape, with dimension of $45 \times 30 \times 30$ Angstroms (Stryer, 1988). ESCA measurement, Figure 4, shows that lysozyme has a large affinity for HOPG. The adsorbed monolayer (the plateau of the adsorption isotherm) was formed within 5 min even when the solution concentration was as low as 0.01 mg/ml, and virtually no desorption was detected.

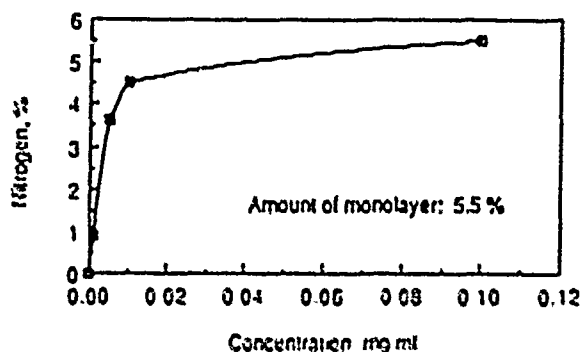


Figure 4. Lysozyme adsorption isotherm detected by ESCA (HF 9958C); adsorption time 5 min at 22°C, and water rinse 1 min.

In Figure 5(a), adsorbed lysozyme is observed by the constant height mode. The molecules apparently collapsed and merged into a rough film. The adsorbate film was apparently thin enough to have avoided being cut through by the STM tip. A very small number of molecules remained roughly of globular shape (Figure 5(b)). But this time their top portions were apparently truncated by the tip. In order to image the whole molecule, the constant current mode should be a better method for molecules with dimensions of more than several Angstroms.

Figure 6 is the image using the constant current mode. Again no individual molecules are recognizable even with higher magnification. Compared with Figure 5(a), this picture shows much rough adsorbates with many "hills" and "valleys". One of the reasons might be that the constant current mode tolerated much larger sized objects since the tip tried to go over them. Another possible reason is that the tip tended to mechanically push the molecules and piled them up if they did not enhance the

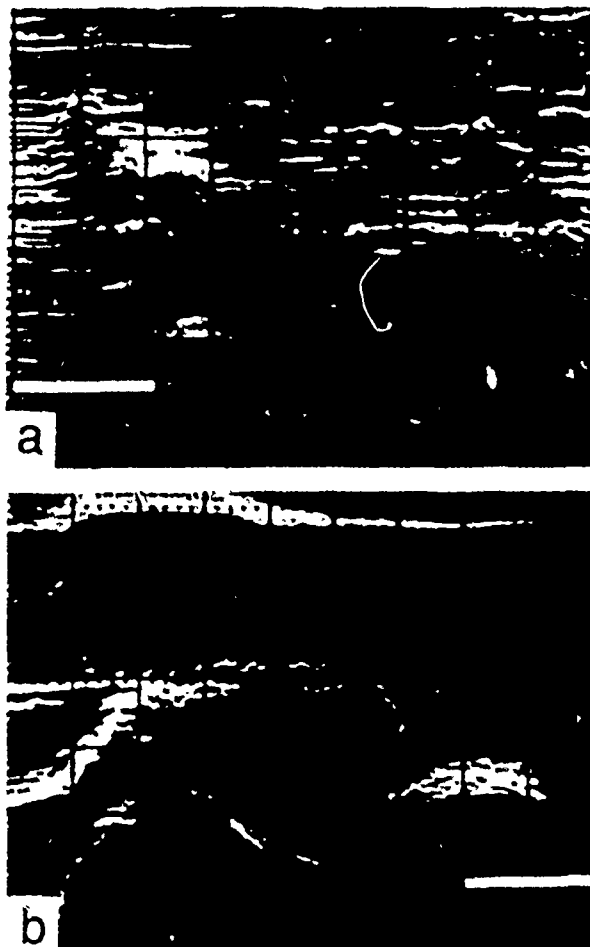


Figure 5. STM images of adsorbed lysozyme on HOPG, adsorption conditions: 0.1 mg/ml concentration, adsorption time 5 min at 22°C, and water rinse 1 min; STM conditions: constant height mode $V_b = 800$ mV, $I_t = 2$ nA. (a) Most lysozyme molecules merged into a rough adsorbed layer, bar = 40 Angstroms. (b) In very few cases globular molecules remained but their top portions were apparently truncated by the tip, bar = 40 Angstroms.

tunneling current sufficiently to provide response in the gap distance adjustment. The latter perhaps dominated since lysozyme molecules had high resistivity and therefore were hardly "seen" by the tip.

Human serum albumin (Feng et al., 1988)

Human serum albumin consists of a single polypeptide chain of 584 amino acids with a molecular weight of about 69,000. Albumin has a strong internal structure, held firmly together by seventeen disulfide bridges. Figure 7



Figure 6. STM images (100 Angstroms/div) of adsorbed lysozyme on HOPG, adsorption conditions: 0.1 mg/ml concentration, adsorption time 5 min at 22°C, and water rinse 1 min; STM conditions: constant current mode, $V_b = 300$ mV, $I_t = 1.8$ nA.

shows the three-dimensional molecular model (Brown and Shockley, 1982). The molecular shape is generally taken as an ellipsoid with dimensions of 40×140 Angstroms. There are three domains within the molecule. The domain structure is believed to be a cylinder formed by six α -helices. This structure has now been partially confirmed by the recent x-ray crystal structure analysis (Carter et al., 1989).

The STM images of human albumin are presented in Figure 8 although they have been difficult to repeat. Figure 8(a) shows one adsorbed human albumin molecule and some parts of two other molecules on HOPG. They have different orientations. The molecular dimensions are 120 Angstroms in length and 61 Angstroms in width. The slight deviation in

dimensions of the adsorbate from those of the above model may be caused by slight collapse of the native structure in the relatively dry air environment. Three cylindrical, parallel domains can be observed, as expected from the model, suggesting that the surface denaturation may not be extensive. In addition to domains, some side loops connecting the domains can also be seen. The fact that domains can be distinguished means that the resolution is about 10 Angstroms. The flat regions around the adsorbed molecules have been identified as bare HOPG. In fact, the tiny ripples along the scanning lines in Figure 8(b) are the corrugation of graphitic carbon atoms, commonly observed by STM on HOPG. The surface depression to the left of each adsorbed molecule is due to the delayed time response of the tip, which was scanning from right to left. Figure 8 gives information on adsorption as well. The albumin molecules essentially maintained a nearly native state in the presence of interactions between the adsorbate and the adsorbent. There has been little apparent denaturation.

Human fibrinogen

The importance of fibrinogen adsorption to the understanding of the blood-compatibility of materials and the molecule's unique three-modular structure lured us to observe it with STM. Fibrinogen is a big protein with molecular weight of 341,000. From the molecular model in Figure 9(a), we see that its dimensions are approximately 450 Angstroms in length and 65 Angstroms in diameter (Williams, 1981). Although we never succeeded in imaging an intact fibrinogen molecule, it turned out that we have ended up with much more information about STM itself.

Figure 9(b) displays a typical fibrinogen molecule, which looks like a "slab". Referring to Figure 9(a), one realizes that the macromolecule observed

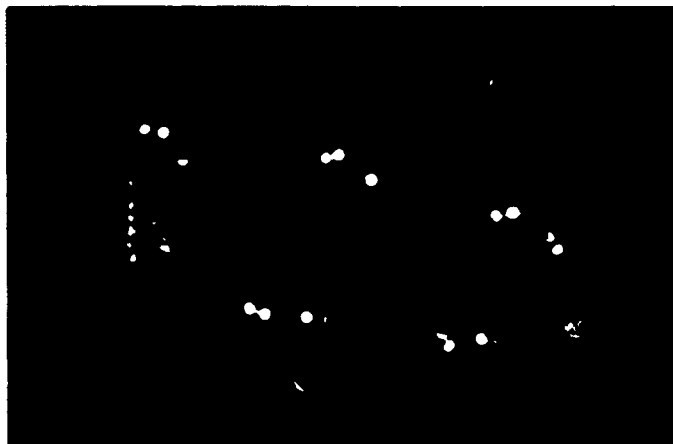
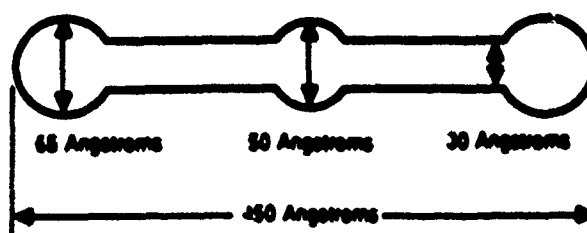


Figure 7. Backbone 3 dimensional model of serum albumin. In this model the wire represents the peptide backbone and the spheres represent disulfide bridges in the long loops. The three domains are antiparallel to one another. The length is 140 Angstroms and the width 40 Angstroms for the entire molecule (from Brown and Shockley, 1982 by permission of the author). See reference.



Figure 8. Images of human albumin on HOPG. The picture dimensions are 200 Angstroms in the horizontal (x) and 160 Angstroms in the vertical (y) directions. Since the adsorbate has a very different electronic structure from the substrate, the height (z direction) could not be directly measured. Both (a) and (b) show several different molecules. Three domains can be clearly seen (a). The ripples on the raster lines in (b) are the corrugations of carbon atoms of the HOPG. The sample was prepared by depositing a droplet of albumin PBS solution (10 ppm albumin) onto freshly cleaved HOPG and then the droplet was removed by capillarity with a tissue. The sample was then flushed with water for 10 sec and was dried at room temperature for 5 h before observation. The STM was operated at a bias voltage of 200 mV, tunneling current of 4 nA and high feedback gain. The constant current mode was used.



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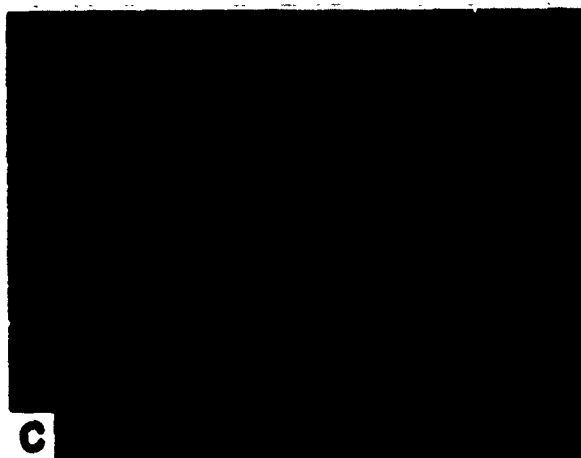
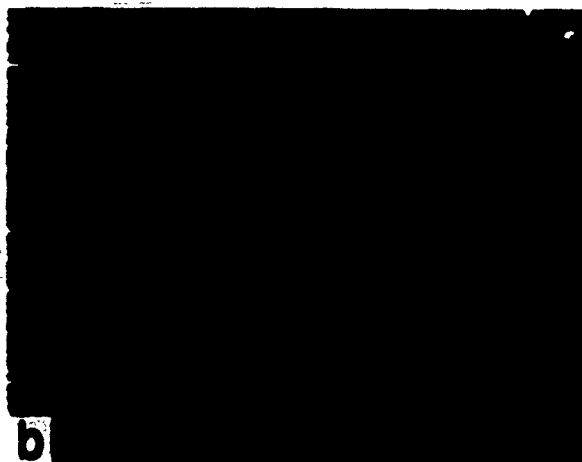


Figure 9. (a) A fibrinogen molecular model from Williams (1981); the globular domains at both ends may be elongated (90 x 40 Angstroms). (b) A typical fibrinogen molecule observed by STM on HOPG (deposited from a 20 ppm aqueous solution), constant current, $V_b = 200$ mV, $I_t = 1$ nA, 100 Angstroms/div. (c) An image of 3 separated domains of a fibrinogen molecule which originally was a similar slab as the one in (a), constant current, $V_b = 200$ mV, $I_t = 1.5$ nA, 100 Angstroms/div. The picture was taken when the trace of water on the sample just disappeared.

in Figure 9(b) had been so severely deformed that it had lost its key characteristics. While its length remained relatively unchanged its width expanded to 400 from 65 Angstroms and its height was reduced to about 15 from 65 Angstroms. The more miserable thing is that it did not show the three domain structure. From the deviation of the molecular dimensions, it is reasonable to suppose that the tip had heavily squeezed, depressed, and thus distorted the fibrinogen molecule. Although fibrinogen has a large molecular size, it appears to not sufficiently increase the tunneling current. Thus, the tip could not discern this huge molecule because it judged the surface morphology by sensing the local tunneling current rather than atomic or molecular topography. Note Figure 9(c): the picture started with a "slab" at the center. A moment later the "slab" suddenly burst into three "caps", probably representing the three deformed node-like domains. This could occur because this sample was just barely dried so the molecule was softer and less adhered and/or denatured. The linking chains between the domains had apparently

been fractured such that the domains were no longer in an axis but rather randomly scattered.

The "slabs" certainly were not part of the substrate, as might be suggested, since they were quite mobile on the substrate. The whole process is illustrated in Figure 10. A single molecule did not have sufficient interaction with the substrate HOPG to immobilize itself. As we mentioned before, owing to some degree of mechanical contact, the tip was driving fibrinogen molecules around and they kept moving until many of them packed together, which increased their mutual interaction. This sort of dynamic process was observed with several different samples.

Although a few individual molecules were imaged, the majority of the STM images showed aggregates of fibrinogen molecules, as in Figure 11, in which the lower left hand flat region was the HOPG substrate. In spite of our attempt to create the conditions favoring the formation of separate molecules, their distribution seems to have nothing to do with the methods of preparation of

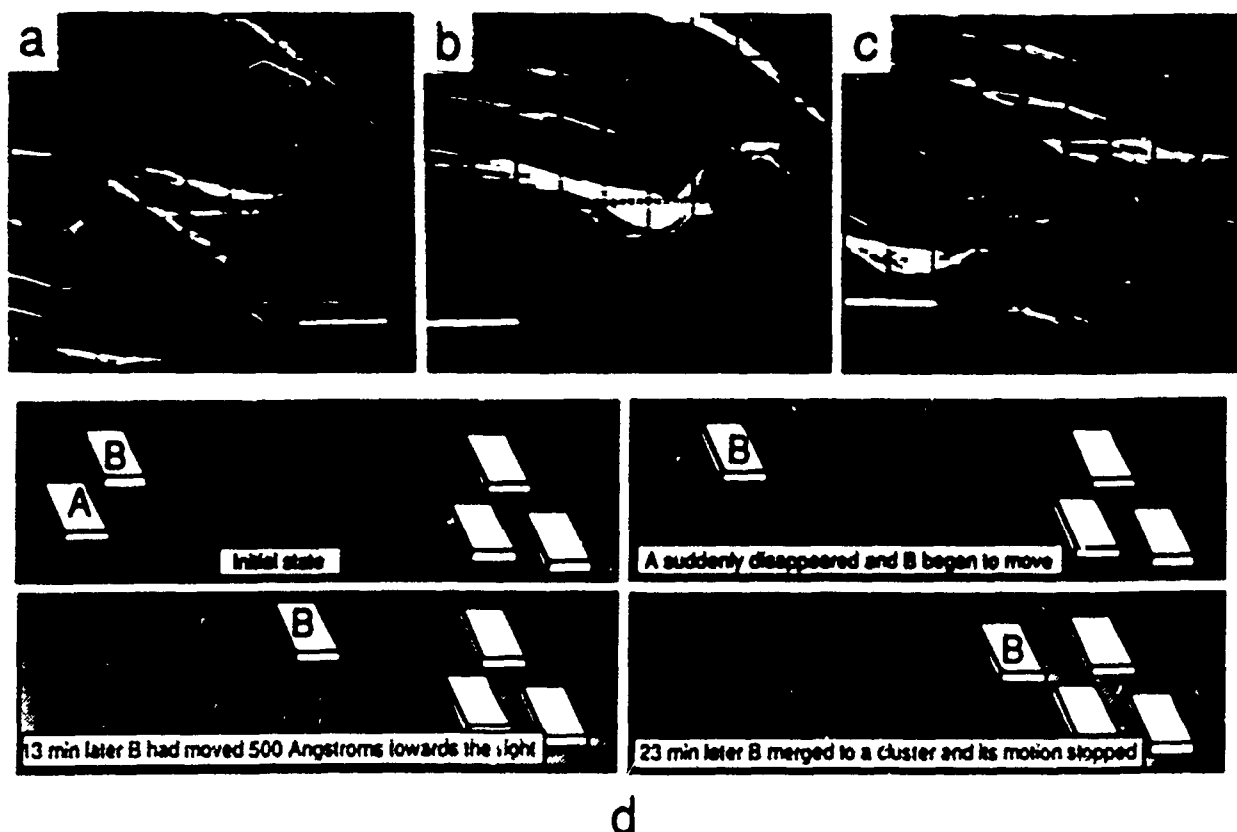


Figure 10. A dynamic process of fibrinogen molecules on HOPG, constant current, $V_b = 200$ mV, $I_t = 3$ nA, 100 Angstroms/div. (a)-(c) recorded the motion and (d) illustrates the entire process.



Figure 11. An edge of adsorbed fibrinogen aggregate, whose area was larger than $1 \times 1 \text{ nm}$. The lower left handed flat region was the HOPG substrate (Deposited from a 5 ppm PBS solution, constant current, $V_b = 300 \text{ mV}$, $I_t = 2 \text{ nA}$, $100 \text{ Angstroms/div}$).

samples, be it deposit or adsorption, whether or not water flashed, and high or low concentrations of the solutions. Just as in the lysozyme case, it is suspected that the tip has driven and piled up segregated molecules into clusters. But this time we have more confidence in this suggestion, since we have evidence of such a dynamic process.

Remarks

Although there is no doubt that protein molecules can be observed on a conducting substrate by STM, depending on various circumstances, the difficulty is how to observe them without altering their initial adsorbed state, how to reproducibly obtain similar images, and under what conditions an adsorbed protein molecule can be unambiguously observed. A number of important questions have to be answered: What is the mechanism of image formation of such poorly conductive substances? What role do the tip geometry and surface chemistry play? What is the major interaction between the tip and a protein molecule: mechanical or electronic? What effects does the conducting substrate impose, such as its deformation, electron density, etc? What is the role of sample hydration or water sorption? We are continuing to address these questions.

Conclusions

Our STM work on proteins adsorbed on HOPG can be summarized as follows:

(1) Both amino acids and proteins can be seen by STM under certain conditions despite some difficulties.

(2) Amino acids are adsorbed both as aggregates and as individual molecules. Single molecules are apt to escape under the STM tip since their interactions with the substrate are weak.

(3) Hen egg-white lysozyme undergoes a conformational change upon adsorption and/or STM visualization.

(4) The high resolution images of human albumin show great promise for STM applications to protein adsorption.

(5) The STM tip may deform human fibrinogen due to mechanical contact, because of its large molecular size and poor conductivity.

(6) Fibrinogen molecules can move over the substrate, driven by the tip, causing them to pile up into clusters.

Acknowledgements

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Discussion with Reviewers

A. Aviram: Can you give a possible explanation of the conduction mechanism of the studied proteins?

Authors: At the present time, there is no general theory which can explain the conduction mechanism of STM images of poorly- or even non-conductive adsorbates. Several hypotheses have been suggested, however, for some particular cases. For example: a) sorbed water may play a role in enhancing the tunneling current; b) near the Fermi level there are some empty states which can relay electrons; c) adsorbates may change the work function of the substrate underneath so that the local environment is different, etc. We did notice the effect of humidity upon the imaging of amino acids and proteins as more of these molecules could be observed in a relatively humid environment than a dry one.

Direct Observation of Immunoglobulin Adsorption Dynamics Using the Atomic Force Microscope

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Abstract

Atomic force microscopic images of a murine antilfluorescyl monoclonal antibody (IgG 4-4-20) depositing from solution onto freshly cleaved mica were observed in real time. These images clearly indicate a cooperative adsorption process, not a random one. Only IgG aggregates formed stable deposits, whereas isolated molecules desorbed readily from the surface. Subsequent adsorption occurred adjacent to the aggregates, forming ridges and eventually a near monolayer was produced. Additional layers deposit only after the initial monolayer adsorption was nearly complete. Desorption of the IgG molecules in a distilled water medium was not observed.

Introduction

Some of the more common methods¹ to study proteins at interfaces are radiolabelling, ellipsometry, total internal reflectance fluorescence (TIRF), infrared (IR), Raman, and X-ray photoelectron (XPS) spectroscopies, and scanning electron microscopy (SEM). While each of these techniques is capable of providing critical information regarding the adsorbed species, this information is actually a measure of the average properties of all the adsorbed proteins in a micron sized (or greater) area. No technique is available with

Langmuir, in press (1989)

which to characterize individual adsorbed proteins. In addition, SEM and XPS are normally used in a vacuum environment which is radically different from an aqueous one in which protein adsorption is occurring. So, the information may not be representative of the actual events. The atomic force microscope (AFM), however, can be operated in an aqueous environment and is capable of providing real time images of protein adsorption with a resolution sufficient to see individual molecules.

The AFM² can be used to obtain atomic scale images of surfaces³⁻⁷. The surface to be imaged is mounted onto a (xyz) piezoelectric crystal and is rastered beneath a sharp tip attached to a cantilever. The tip rides across the surface and the forces between the surface and the tip cause deflection of the cantilever. This deflection can be monitored using a scanning tunneling tip^{2,3} or more easily, it can be monitored by movement of a laser beam that is reflected off the back of the cantilever^{8,9}. Since the cantilever is sensitive to the intermolecular forces between the tip and the surface, the sample need not be a conductor to be imaged. Images have not only been obtained from graphite³⁻⁵ and metals⁶, but also from semiconductors^{4,7} and insulating polymers¹⁰⁻¹². Magnetic fields¹³ and charged regions in materials¹⁴ have been imaged as well. More detailed reviews of the AFM theory are presented by Marti et al. and Hansma et al.^{15,16}.

The AFM has already been used to image surfaces in an aqueous environment¹⁷. Underwater images of crystalline mica and polyalanine on mica have been obtained. One advantage of using water as a scanning medium is the minimization of general adhesion forces that result between the tip and the surface¹⁸. Such forces dominate the interaction between the tip and the sample and prohibits the possibility of obtaining high-resolution images. In addition, scanning surfaces in aqueous environments enables one to realistically image

biological systems. The AFM can obtain new images within a few seconds and can therefore monitor biological processes in real time. Recently, Hansma et al.¹⁷ were able to follow the formation of a polymerized fibrin network on a mica surface by adding thrombin to a solution of fibrinogen. These images showed fibrin oligomers aggregating to form a single polymer strand. Formation of additional strands occurred adjacent to first.

This paper discusses the images obtained from the adsorption of a murine anti-fluorescyl monoclonal immunoglobulin G (4-4-20 IgG₂ (κ))¹⁹ from solution onto clean mica surfaces. This protein was chosen because it is easily crystallized and has self-aggregating properties. We hoped that some unique ordering upon adsorption to the mica surface might occur and, if so, this ordering could be imaged with the AFM. We felt that desorption could be observed as well.

Methods

The AFM experimental apparatus has already been described and can be found elsewhere¹⁶. Movement of the microfabricated cantilever²⁰ is detected by the positioning of a laser light beam that has reflected off the back of the cantilever and is detected by a pair of photodiodes. The AFM images are continuously recorded on video tape for later review. A flow cell has been set-up across the surface of the mica that allows rapid exchange of the fluid.

Mica (Asheville-Schoonmaker) was affixed to the piezoelectric crystal stage and cleaved *in situ*. The flow cell was constructed around the stage and distilled water was injected onto the mica surface. The microcantilever was advanced until the force between the tip and the surface approximated 10^{-9} N. The mica was then imaged continuously in the feedback mode with a scan area of 1800 \AA by 1800 \AA and a constant scan speed of 16 msec/line.

The AFM tip was retracted from the mica surface and a solution of 18 $\mu\text{g/mL}$ IgG 4-4-20 (a gift from J. N. Herron) in phosphate buffered saline (pH 7.4) was injected into the flow cell. The tip was advanced to the mica surface which was then imaged continuously in the feedback mode over a scan area of 1800 Å by 1800 Å. After 4 minutes, the scan area was increased to 4500 Å by 4500 Å (full scale). After another 1 1/2 minutes, the AFM was switched to variable force mode for the remainder of the imaging.²¹ The adsorption process was imaged continuously for 40 minutes. During this time, the scan area was decreased to 1800 Å by 1800 Å, to 900 Å by 900 Å, and then returned to full scale.

Immediately after the 40 minute IgG adsorption, the tip was retracted and the flow cell was flushed with distilled water. The surface was scanned for 10 minutes at full scale, at 1800 Å by 1800 Å, and then at 900 Å by 900 Å.

Results

The image obtained of the mica surface underwater is flat and featureless indicating a pristine surface.

Within the first two minutes after injection of the IgG into the flow cell, a continuously growing aggregate was observed in the lower right-hand corner of the screen. This image was obtained in feedback mode and had dimensions of 1800 Å by 1800 Å. This aggregate appeared on top of the featureless mica background. After five minutes, the scan area was increased to 4500 Å by 4500 Å and 'ridges' appeared (A). The AFM was then switched to variable force mode (B) and the same image appeared (different contrast) indicating that either mode could be used. As time progressed, it was clear how the adsorption was taking place. Molecules that landed adjacent to these ridges would adhere resulting in two-dimensional growth in the plane of the surface (C). Yet most molecules that landed by themselves would desorb readily as evidenced by the disappearance of these isolated molecules. The size of these molecules

roughly matched the known size of an IgG molecule. The deposited IgG appeared as mounds and subsequent frames showed smearing of these images.

The ridges continued to spread (D,E) along the surface until a monolayer covered the surface of the mica. Although it is difficult to obtain accurate height dimension in variable force mode, the monolayer thickness was approximated at 50 Å, which is consistent with the dimensions of an IgG molecule. Near the end of the monolayer formation, a second layer started to appear. This second layer arose from many different sites on the first layer since protein interactions could occur from anywhere on the surface (F). Upon growth of the second layer, most IgG molecules that deposited would adhere, but then smear, suggestive of a rapid conformational change.

After the water flush, the surface exhibited altered features, but there was no evidence that IgG desorption was taking place.

Discussion

The observations of deposited IgG on the surface appearing only as aggregates and of individual mounds rapidly desorbing from the surface are suggestive of lateral interactions occurring between the adjacent IgG molecules, which appear to be important for formation of a stable protein layer. These lateral interactions are not unexpected, since this protein has some self-aggregating properties. What is interesting is the necessity of lateral interactions for adherence to the surface. Perhaps, an IgG molecule by itself can only get a toe-hold on the surface at first and can be desorbed easily. Once it has multiple holds with the surface and neighboring molecules, the probability of desorption decreases significantly¹. This would explain the phenomena we observed here. This argument is also supported by the observation of a second IgG layer arising from many different areas on the monolayer surface. Here, the deposited

IgG can arise from any number of places, since interactions can occur from anywhere on the monolayer surface. Prior to this experiment, a protein adsorption isotherm on mica was obtained using ^{125}I -labelled IgG. The isotherm showed Langmuir-like behavior and at a concentration of $18\text{ }\mu\text{g/mL}$, the mica surface was only 40% covered (less than a monolayer).

After the water flush, IgG desorption was not observed. Perhaps, desorption was slower than the observation time (minutes) due to the strength of interaction between the adjacent IgG molecules. Previous data has shown²² that IgG does desorb from a silica surface, but that study was performed using polyclonal IgG and may behave differently from the present system.

Certainly, these observations were not solely a consequence of simple adsorption phenomena. A number of times, protein mounds would be displaced parallel to the rapid scanning direction only to be returned to its original position upon subsequent images. It is clear that the tip of the probe is 'massaging' or pushing the molecules on the surface. The extent to which this occurs, however, is not known and any conclusions can only be made keeping this in mind. For example, maybe the formation of aggregates results from the probe pushing the molecules over to a small cluster of molecules. The probe may not be able to displace this cluster laterally because of the strength of interaction it has with the surface and can only 'hop' over it. In this manner, the probe may behave as a gathering device which sweeps the molecules into piles. This phenomena may explain the discrepancy between the AFM images and the isotherm data. In addition, the desorption of individual molecules from the surface may be a result of tip interaction. Thus, the probe may sweep these molecules off the surface as well.

The authors would like to emphasize that this experiment is a preliminary one done at one protein concentration. The effect of concentration on the

adsorption pattern is yet unknown, but is the subject of ongoing studies. Furthermore, the issue of protein adsorption on the probe itself was not addressed here. This issue is important and merits further detailed study and consideration.

Conclusion

Using an atomic force microscope, real time imaging of IgG deposition on flat mica was accomplished. While IgG adsorption may occur anywhere on the surface, only those molecules with sufficient lateral interactions had the capability to remain on the surface. Isolated molecules desorb readily. A second layer could be observed after 35 minutes of adsorption. It was hoped that unique ordering of the IgG on the surface could be visible, but this was not evident. Although, restructuring of the surface had occurred in the desorption experiment, desorption was not conclusive.

While it is exciting that individual molecules could occasionally be seen, it is not clear how much the probe affects molecular conformation. When more sensitive cantilevers and more sophisticated detection systems are developed, it may be possible to operate the AFM using forces of 10^{-10} to 10^{-11} N and image biomolecules unperturbed by the probe.

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Image A. Feedback image of 18 $\mu\text{g/mL}$ IgG 4-4-20 in PBS on clean mica after 5 minutes. Note the formation of aggregate 'ridges'. The scan area is 4500 Å by 4500 Å.

Image B. Variable force mode image of 18 $\mu\text{g/mL}$ IgG 4-4-20 in PBS on clean mica after 5 1/2 minutes. The ridges are now resolved better. The scan area is 4500 Å by 4500 Å.

Images C,D,E. Variable force mode images of 18 $\mu\text{g/mL}$ IgG 4-4-20 in PBS on clean mica. Times of adsorption are 15, 17 1/2, and 20 minutes. Growth of the monolayer centers about the ridges and proceeds until a near monolayer is formed. The scan areas are 4500 Å by 4500 Å.

Image F. Variable force mode image of 18 $\mu\text{g/mL}$ IgG 4-4-20 in PBS on clean mica. Adsorption time is 37 minutes. After a near complete monolayer is formed, a second layer begins to deposit. The scan area is 4500 Å by 4500 Å.



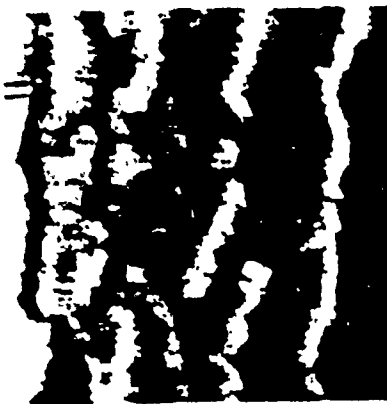
A



B



C



D



E



F

REAL TIME IMAGING OF IMMUNOGLOBULIN ADSORPTION ON MICA USING THE ATOMIC FORCE MICROSCOPE

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Introduction

There are a number of methods to study the adsorption of proteins at interfaces including radiolabelling, scanning electron microscopy, and a variety of spectroscopic techniques. The problem with these methods is that it is not possible to image the molecular arrangement of adsorbed molecules, let alone individual molecules on a surface. Furthermore, some of these techniques can not monitor the adsorption events in real time or in an aqueous environment. The advent of the atomic force microscope (AFM) has enabled researchers to monitor real time processes in an aqueous environment. Recently, the AFM community at UCSB was able to image the adsorption and polymerization of fibrinogen on mica demonstrating the potential of the AFM for observing biological processes.¹

With an invitation to use the AFM instrumentation at UCSB, those of us at Utah decided to study the adsorption of a monoclonal IgG (4-4-20) on mica.² This protein was chosen because it is easily crystallizable and was therefore expected to display non-random adsorption.

Methods

Mica was attached onto the piezoelectric crystal and a fresh surface was produced by cleaving *in situ*. A flow cell was erected around the mica and distilled water was introduced. The piezoelectric stage was advanced until the force between the AFM tip and the sample approximated 10^{-9} N. Images of the mica surface were thus obtained. After tip retraction, the distilled water was exchanged with an 18 mg/mL solution of IgG (4-4-20) in phosphate buffered saline (pH 7.4). Images obtained showed the progression of IgG adsorption dynamics. IgG desorption in distilled water was investigated as well.

Results and Discussion

Images were obtained in both feedback and variable force modes over a typical scan area of 4500 \AA by 4500 \AA . The image of the mica was featureless indicating a flat, virgin surface.

Images from the IgG solution experiment showed adsorption was a cooperative process. The first images were obtained in feedback mode and showed a growing IgG aggregate. The scan area here was 1800 \AA by 1800 \AA . After 5 minutes from the time of protein addition, the scan area was increased to 4500 \AA by 4500 \AA and 'ridges' appeared. The scanning mode was switched to variable force mode and the same image appeared. Rarely were isolated molecules seen and those that were observed disappeared by the next image (5 seconds later). Yet, those that landed adjacent to the ridges adhered resulting in growth in the plane of the surface. These observations indicate that lateral interactions are necessary for a stable protein layer on mica. The adsorbed layer continued to grow in two dimensions producing a near complete monolayer at 20 minutes. At 30 minutes, a second IgG layer depositing on the monolayer becomes visible. No desorption of the IgG layer in distilled water was observed, even after 10 minutes, perhaps due to the strength of lateral interactions.

Since individual IgG molecules were not observed in these images, we are now trying to obtain images of an easily crystallizable IgM pentamer. Due to its symmetry and larger size, we hope to be able to see individual molecules.



Image 1: Variable force mode image of 18 mg/mL IgG 4-4-20 in PBS adsorbing on mica after 5 1/2 minutes. Scan area is 4500 \AA by 4500 \AA . Note formation of 'ridges'.

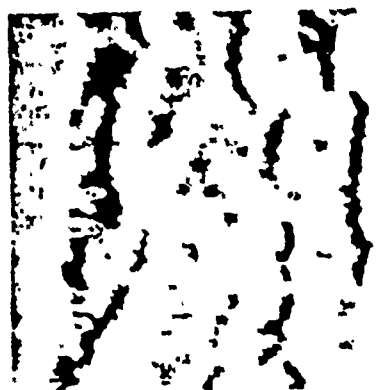


Image 2: Variable force mode image of 18 mg/mL IgG 4-4-20 in PBS adsorbing on mica after 20 minutes. Scan area is 4500 \AA by 4500 \AA . A monolayer is nearing completion.

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